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Production of Red Pigments by *Monascus purpureus* in Submerged Culture

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Abstract For the purpose of mass producing *Monascus* red pigments optimum medium composition and environmental conditions were investigated in submerged flask cultures. The optimum carbon and nitrogen sources were determined to be 30 g/L of glucose and 1.5 g/L of monosodium glutamate (MSG). Of the three metals examined, Fe^{2+} showed the strongest stimulatory effect on pigment production and some stimulatory effect was also found in Mn^{2+}. Optimum pH and agitation speed were determined to be 6.5 and 700 rpm, respectively. Under the optimum culture conditions batch fermentation showed that the maximum biomass yield and specific productivity of red pigments were 0.20 g DCW/g glucose and, 32.5 OD_{500} g DCW^{-1} h^{-1}, respectively.

Keywords: *Monascus purpureus*, red pigments, submerged culture, nutritional effect

INTRODUCTION

Since many kinds of synthetic dyestuffs have been found to be hazardous to human health, only limited kinds of such dyestuffs are permitted to be used in food in many countries, and therefore, there is a need to develop alternative sources of natural food colorants.

There are a number of natural colorants, but only a few available in sufficient quantity are of industrial use because they are directly extracted from plant flowers, fruits, leaves and roots [1]. It is therefore advantageous to produce natural coloring agents from microorganisms, and some pigments have been produced from *Monascus* [2,3], *Streptomyces* [4], and *Serratia* [5].

Pigments synthesized by the fungi *Monascus* spp. have been traditionally used in Asia for colouring and securing a number of fermented foods [6]. Furthermore, their therapeutic properties and their relatively high stability with respect to pH and temperature are interesting features that promote their use as substitutes for synthetic colorants [7]. *Monascus* can produce at least six major related pigments [8], which may be divided into three groups: two are orange (rubropunctain and monascoubrin), two are yellow (monascin and ankaflavin) and two are red (rubropunctaminea and monascorubramine). Among these, the red pigments are of particular interest, because red is the most popular food color and true red natural pigments suitable for food use are difficult to obtain [9].

There have been reports that *Monascus* can be cultured in submerged culture system [3,6,9,10], which could overcome the drawback of low yield and its large area requirement of the solid-state culture system.

In this study, to improve the production of *Monascus* pigment, many factors such as carbon and nitrogen sources, pH, and minerals that influence pigment production were evaluated in submerged flask cultures. Batch fermentations were performed under optimum culture conditions obtained from submerged flask cultures.

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MATERALS AND METHODS

Microorganism

The strain used in this study was *Monascus purpureus* (ATCC 16365). The stock culture was maintained on YM agar slant [11] containing: glucose, 20 g; malt extract, 3 g; peptone, 5 g; yeast extract, 3 g; agar, 1.5 g; and distilled water, 1 L.

Media

Seed culture was conducted in YM medium, and submerged flask culture was performed using Lin's medium, which contains rice powder, 30 g; $NaNO_3$, 1.5 g; KH_2PO_4, 2.5 g; $MgSO_4 \cdot 7H_2O$, 1.0 g per liter of distilled water. The initial pH of the medium was adjusted to 6.5 and was not regulated during flask culture. Batch fermentation was conducted using modified Lin's medium, which was optimized in the flask cultures (Table 1). The medium was sterilized at 121°C for 15 min. Before autoclaving, the pH of the medium was adjusted to 7.0.

Cultivation

The seed culture was performed, with 5 pieces of agar

Table 1. Composition of the modified Lin's medium

Component	Concentration
Glucose	30.0 g/L
MSG	1.5 g/L
KH_2PO_4	2.5 g/L
$MgSO_4 \cdot 7H_2O$	1.0 g/L
$FeSO_4 \cdot 7H_2O$	14.0 mg/L

disk (3 × 4 mm) taken off a solid agar plate on which *Monascus* was grown, in a 250-mL flask with 100 mL working volume at 30°C, and 150 rpm for 4 days. A 5% volume of seed culture, which was made uniform using a homogenizer, was used as its inoculum for the flask culture. In order to investigate the effect of carbon and nitrogen sources on cell growth and red pigment production the flask cultures were carried out in a 250-mL flask containing 100 mL medium with various carbon and nitrogen sources at 30°C, and 150 rpm for 4 days. The effects of trace elements and pH were also tested. Batch fermentations were performed in a 3-L bioreactor (BioFloIII, USA) with a working volume of 1.5 L at 30°C for 4 days; its stirring rate between 400 and 900 rpm, and its aeration rate was 1.0 vvm. The percentage of dissolved oxygen was maintained above 20%. The pH was controlled at 7 by adding 2 N HCl and 2 N NaOH, and foaming was controlled using antifoam A (Sigma).

Assays

Twenty mL of culture broth was collected aseptically and filtered through a Whatman No. 1 filter paper, and washed twice with 10 mL of distilled water. The biomass was determined by weighing the paper after drying the mycelia at 80°C for 24 h. The filtrate was used to estimate the extracellular red pigments using a HP 8890 UV/VIS spectrophotometer at 500 nm. Uninoculated medium was used as a blank. To estimate the concentration of its intracellular pigments, 20 mL of culture was filtered and washed with distilled water. Washed mycelia were extracted with 20 mL of 95% ethanol for 24 h with continuous agitation. The concentration of red pigments was determined from the absorbance of the extract at 500 nm. The results for red pigment production were expressed as the absorbance units (U) multiplied by a dilution factor. Glucose was determined using the dinitrosalicylic acid method [12].

RESULTS AND DISCUSSION

Effect of Nutrients and pH on Cell Growth and Red Pigment Production

Effect of Carbon Source

The effect of different carbon sources at a concentration of 30 g/L on cell growth and pigment production was investigated in flask cultures. As shown in Fig. 1, *M. purpureus* cells used almost all of the substrates tested as

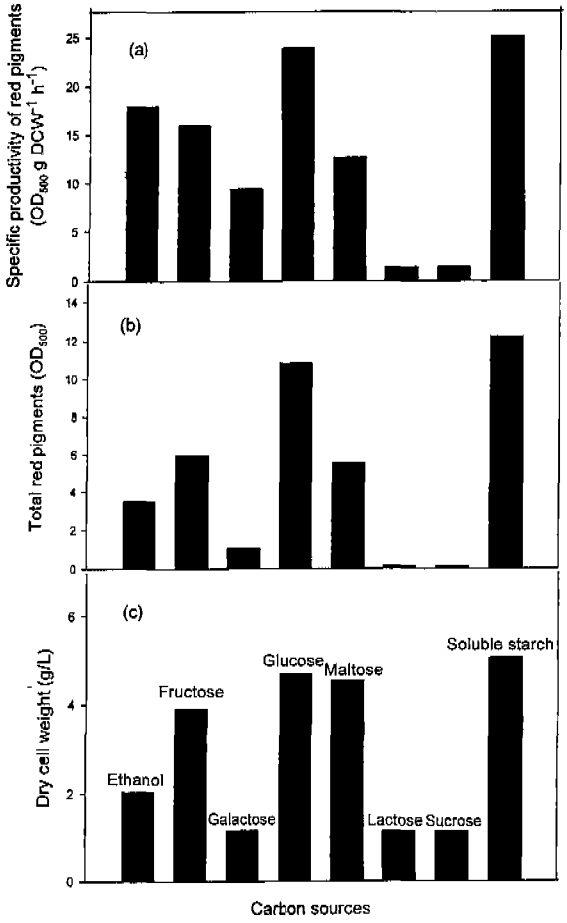

Fig. 1. Effect of carbon sources on cell growth and red pigment production: (a) specific productivity of red pigments, (b) total red pigments, and (c) dry cell weight.

carbon sources for cell growth and pigment production. Among these, glucose and soluble starch proved to be better than other carbon sources in terms of both growth and pigment production. The majority of authors have reported that glucose is a superior substrate for pigment production by *Monascus* species [11,13]. Soluble starch may make the culture viscous and interfere with the aeration of the cultures, restricting the amount of dissolved oxygen. Therefore, glucose was determined to be its best of the carbon sources tested. Little pigment production was observed during a 4-day experiment with lactose and sucrose.

When growth and pigment production were examined at different glucose concentrations (5-50 g/L), maximum specific productivity of red pigment was obtained at a glucose concentration of 30 g/L (Fig. 2). Up to 30 g/L, increasing glucose concentration increased both biomass and pigment production, but above this concentration biomass and pigment production were drastically reduced due to Crabtree effects, which generally occur in yeast batch fermentation with high sugar concentrations and inhibit respiratory enzyme

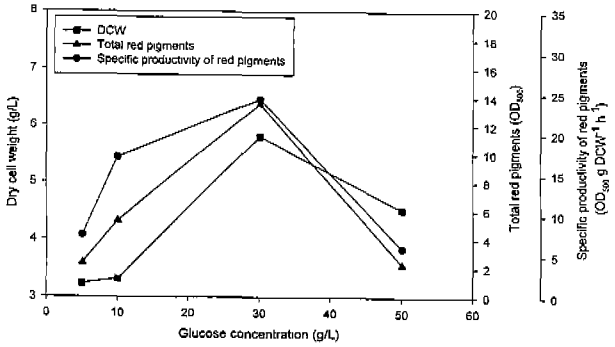

Fig. 2. Effect of glucose concentration on cell growth and red pigment production.

and increase ethanol production [14]. Chen and Johns also reported that a high glucose concentration (50 g/L) led to low growth rates, pigment synthesis and considerable ethanol production [9,10]. The glucose when consumed was first metabolited to acetyl CoA, which could be channelled into pigment production on entry to the TCA cycle [8]. This suggests that the *Monascus* pigment could serve as a carbon sink, by incorporating the carbon of acetyl CoA when glucose is present in excess, but in our experiment this phenomenon was not found.

Effect of Nitrogen Source

The effect of nitrogen source on cell growth and pigment production was studied in flask cultures with various nitrogen sources, since nitrogen sources have been found to have a great effect on the quality and quantity of *Monascus* pigments produced [15]. The results obtained are shown in Fig. 3. Ammonium nitrate, sodium nitrate and monosodium glutamic acid (MSG) showed good results upon pigment production, but produced less biomass than other nitrogen sources, except ammonium sulfate. Of all the nitrogen sources tested MSG gave the highest specific productivity of red pigments. Lin also reported that MSG was the most favorable nitrogen source for the formation of red pigment [6], but there still exists some controversy over the best nitrogen source for red pigment production with organic nitrogen [3,16] and nitrate [6,17] being favored. As shown in Fig. 3, a high concentration of biomass was obtained with soybean and yeast extract, whereas the specific productivity of red pigments was reduced to about a third of that obtained with MSG. Juzlova have also reported yeast extract stimulated conidiation, repressed the sexual cycle and increased biomass production [18]. Ammonium chloride gave some different result from the report that ammonium chloride was a better inorganic nitrogen source than sodium nitrate for biomass and pigment production [9,10]. The utilization of nitrogen sources for pigment production appears to be strain-specific since other strains produce much more pigment with MSG and sodium nitrate [3,6].

The effect of MSG concentration on cell growth and

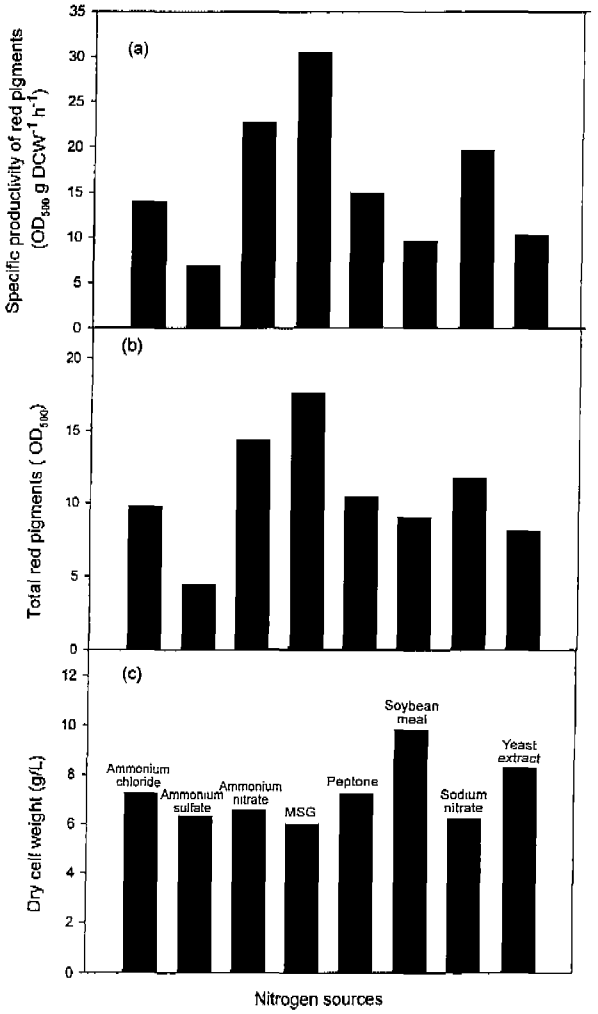

Fig. 3. Effect of nitrogen sources on cell growth and red pigment production: (a) specific productivity of red pigments, (b) total red pigments, and (c) dry cell weight.

pigment production is shown in Fig. 4. Optimum MSG concentration was determined to be 1.5 g/L by flask culture at various concentrations of MSG. Increased MSG concentrations increased biomass, but concentrations of over 1.5 g/L of MSG gave increased biomass and decreased pigment production

Effect of Trace Metals

Flask cultures were performed using trace metals including Zn^{2+}, Mn^{2+}, and Fe^{2-} to investigate the effect of each metal on cell growth and pigment production and the results are shown in Fig. 5. Of these three metals, Fe^{2+} showed the strongest stimulatory effect on red pigment production. In the case of Zn^{2+}, Bau and Wong [19] reported that pigment production by a mutant *M. purpureous* N11S was promoted by 5×10^{-5} M of zinc while mycelial growth was significantly inhibited. However, its relationship was reversed in our experiment. This result may be attributed to strain specificity.

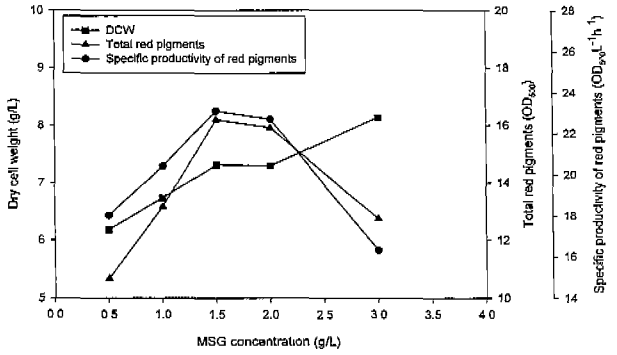

Fig. 4. Effect of MSG concentration on cell growth and red pigment production.

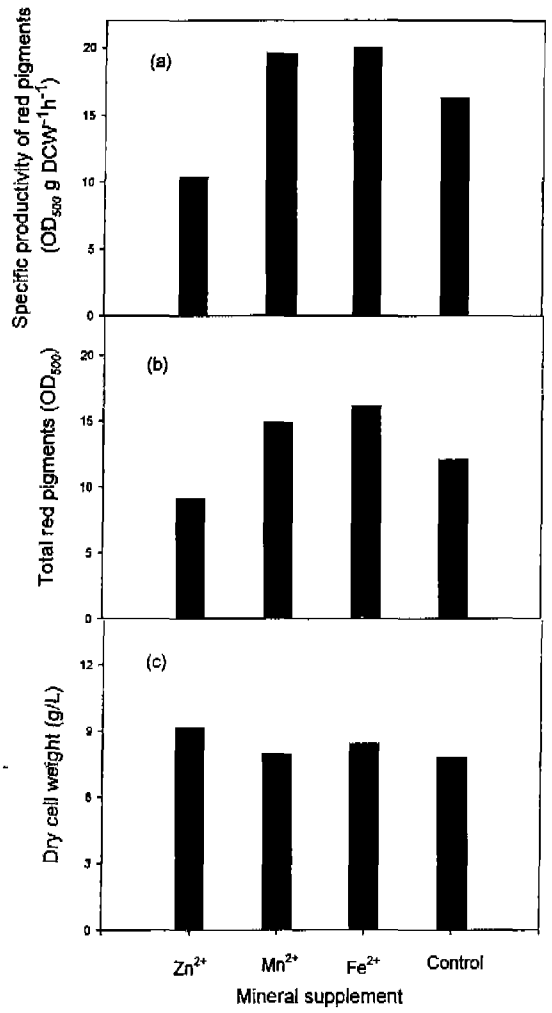

Fig. 5. Effects of minerals on cell growth and red pigment production: (a) specific productivity of red pigments, (b) total red pigments, and (c)dry cell weight.

Some stimulatory effect has also been attributed to Mn^{2+}, because trace metals have important effects on secondary metabolism [20]

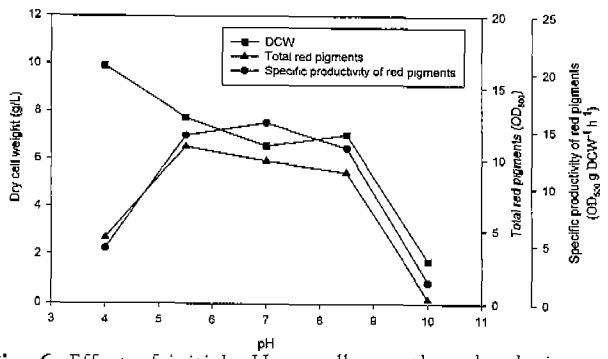

Fig. 6. Effect of initial pH on cell growth and red pigment production.

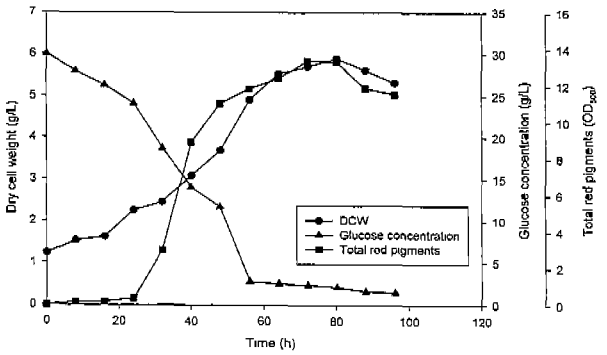

Fig. 7. Mycelial growth and red pigment production profiles versus time in batch fermentation under the optimum culture conditions.

Effect of pH

The results from flask cultures performed using optimized medium at various pH are presented in Fig. 6. The initial pH value of the medium markedly influenced red pigment formation. A pH range of 5.5 to 8.5 favored red pigment production. At pH higher than 8.5 and lower than 5.5 red pigment production decreased noticeably. Maximum biomass and red pigment production were obtained at pH 4.0 and 7.0, respectively. On the other hand, Carels and Shepherd [17] reported that reducing the pH inhibits the formation of conidia and increases pigment production, suggesting that pH of the medium might affect the transport of certain media constituents such as glucose and nitrogen sources. Growth and activity of fungus was better at pH 4.0 than at pH 7.0. This was evidenced by markedly higher maximum specific rates of glucose consumption and faster specific growth rate at pH 5.5 (data not shown). Regardless of the initial pH, the final pH of the cultures showed a similar value. This pH change during cultivation depends on the nitrogen source [17] and, to lesser extend, on the carbon source [20].

Batch Fermentation

Batch fermentations were performed using a modified Lin's medium, which was optimized during our

experiment and gave the highest pigment production in the submerged flask culture experiment. Optimum agitation speed was determined to be 700 rpm in batch fermentation (data not shown). In submerged mold culture, mycelia may grow into the homogeneous and filamentous suspensions (so called pulp type growth), the globeose colonies (so called pellet type growth), or the intermediate forms between pulp and pellet type [21]. It was reported that cultivation at optimal agitation speed produces mycelial forms of intermediate type and provides an effective means of increasing the pigment yield [22]. At a speed of 700 rpm, the similar results were found that cells showed the intermediate forms between the pulp and pellet type, and pigment production was good. At a agitation speed of 900 rpm, pellet type mycelia predominated and pigment production was drastically decreased. It is believed that violent agitation caused an undesirable effect on cell growth and pigment production due to high shear stress. Fig. 7 shows the growth and pigment production profile versus time in a batch fermentation under optimum culture conditions. As shown in Fig. 1, *M. purpureous* depleted glucose after 4 days incubation, and produced 5.90 g/L of biomass. Red pigment production started at the beginning of the exponential growth phase, increased sharply after 30 h, and reached 13.37 OD_{500} at the stationary phase. Other workers have also reported this growth associated pigment production by *Monascus* [23]. However, it is not believed that the polyketide pathway leads exclusively to secondary metabolite [24]. At the late log phase the pigment production rate showed a little retardation. *Monascus* pigments are polyketides and oxygen is an essential substrate for their biosynthesis [24]. It is possible that the final high viscosity of the culture broth in the late log phase interfered with the aeration of the cultures. Some pigment loss was observed in the stationary phase from the absorbance measurement of pigment concentration. It is thought that an enzymatic pathway, which may be induced by nutrient exhaustion, degrades the pigment. Enzyme degradation of secondary metabolites is a common phenomenon in fungi [25]. The maximum biomass yield and specific productivity of red pigment were 0.20 g DCW/g glucose and, 32.5 OD_{500} g DCW^{-1} h^{-1}, respectively.

CONCLUSION

Monascus red pigment production was highly dependent on nutritional and environmental conditions. Among the 8 kinds of carbon and nitrogen sources examined, glucose and MSG were determined to be the best carbon and nitrogen source, respectively. Maximum specific productivity of red pigment was obtained at 30 g/L of glucose and 1.5 g /L of MSG. Of the three metals examined, Fe^{2+} showed the strongest stimulatory effect on pigment production and some stimulatory effect was attributed to Mn^{2+}. However, zinc inhibted red pigment production. The pH range of 5.5 to 8.5 favored the production of red pigment, and at pH higher than 8.5 and lower than 5.5 red pigment production decreased noticeably. In batch fermentation, the optimum agitation speed was determined to be 700 rpm. At optimum culture conditions batch fermentation showed that the maximum biomass yield and specific productivity of red pigment were 0.20 g DCW/g glucose and, 32.5 OD_{500} g DCW^{-1} h^{-1}, respectively.

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